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Arhgef16, a novel Elmo1 binding partner, promotes clearance of apoptotic cells via RhoG-dependent Rac1 activation

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ABSTRACT

Elmo is an evolutionarily conserved mammalian ortholog of *Caenorhabditis elegans* CED-12 with proposed roles during the removal of apoptotic cells, cell migration, neurite outgrowth, and myoblast fusion (Katoh and Negishi (2003) [1], Park and Tosello (2007) [2], Grimsley et al. (2004) [3], Hamoud et al. (2014) [4]). Elmo mediates these cellular processes by interacting with various proteins located in the plasma membrane, cytoplasm and nucleus, and by modulating their activities although it has no intrinsic catalytic activity (Park and Tosello (2007) [2], Hamoud et al. (2014) [4], Li et al. (2013) [5], Margaron, Fradet and Cote (2013) [6], and Mauldin et al. (2013) [7]). Because there are a limited number of proteins known to interact with Elmo, we performed a yeast two-hybrid screen using Elmo1 as bait to identify Elmo1-interacting proteins and to evaluate their mode of regulation. Arhgef16 was one of the proteins identified through the screen and subsequent analyses revealed that Arhgef16 interacted with Elmo1 in mammalian cells as well. Expression of Arhgef16 in phagocytes promoted engulfment of apoptotic cells, and engulfment mediated by Arhgef16 increased synergistically in the presence of Elmo1 but was abrogated in the absence of Elmo1. In addition, Arhgef16-mediated removal of apoptotic cells was dependent on RhoG, but independent of Dock1. Taken together, this study suggests that the newly identified Elmo1-interacting protein, Arhgef16, functions synergistically with Elmo1 to promote clearance of apoptotic cells in a RhoG-dependent and Dock1-independent manner.

1. Introduction

Clearance of apoptotic cells in multicellular organisms is important for preventing secondary necrosis and inflammation and removal of apoptotic cells is critical for maintaining tissue homeostasis and for regulating immune responses [8–10]. Elmo, one of the key proteins involved in this process, is a mammalian homolog of CED-12 in *Caenorhabditis elegans* and is evolutionarily conserved from worms to mammals. Elmo is known to be involved in various cellular processes such as phagocytosis, cell migration, neurite outgrowth, and formation of cellular protrusions via cytoskeleton remodeling [1,2,5,6,11,12]. Interestingly, Elmo regulates these processes by modulating the activities of other proteins and by serving as a scaffold for transferring signals to other downstream molecules through protein–protein interactions despite lacking any intrinsic catalytic activity [1,12–15].

One well-known Elmo-interacting protein whose activity is regulated by Elmo is Dock1. Dock1 is an unconventional guanine nucleotide exchange factor (GEF) for Rac because it has a docker domain in place of the Dbl homology domain found in conventional GEFs [13,16]. It has been proposed that Elmo can regulate the GEF activity of Dock1 by several different mechanisms: the N-terminus of Elmo targets the Elmo–Dock1 complex to the plasma membrane where the complex has easy access to Rac1; Elmo also relieves the self-inhibitory state of Dock1 caused by the binding of the SH3 domain to the docker domain via the interaction between the PXXP motif of Elmo1 and the SH3 domain of Dock1; finally Elmo helps Dock1 to stabilize nucleotide-free Rac1 in the transition state. Thus, Elmo helps to modulate the GEF activity of Dock1 by a variety of mechanisms [12,14,15].

In addition to Dock1, a few other proteins that interact with Elmo have been identified. RhoG is a member of the Rho family of small

Abbreviations: Elmo, engulfment and cell motility; GEF, guanine nucleotide exchange factor; IP, immunoprecipitation; GST, glutathione S-transferase; AG16, Arhgef16; E1, Elmo1; D-ISP, Dock1-ISP; DC, dendritic cell; TEC, thymic epithelial cell; PEC, peritoneal exudate cell; BM, bone marrow; Spln, spleen; Thy, thymocytes; MEF, mouse embryonic fibroblast; Cont, control; KD, knockdown; ppt, precipitation; RT-PCR, reverse transcriptase-polymerase chain reaction.

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GTPases and is involved in cellular morphological processes such as neurite outgrowth in neuronal cells, which requires the activation of Rac1. The GTP bound form of RhoG, but not the inactive form, specifically binds to the N-terminus of Elmo. The C-terminus of Elmo binds to Dock1 simultaneously to form a ternary complex capable of activating Rac1. Thus, Elmo is a mediator that links RhoG activation to Rac1 by interacting with both RhoG and Dock1 [1,17,18].

Another interesting Elmo-interacting protein, BAI1, is a member of the class of adhesion G-protein coupled receptors (GPCRs) which functions as an engulfment receptor for apoptotic cells upstream of the Elmo–Dock1–Rac module. Once apoptotic cells are recognized by the TSR domain in the extracellular region of BAI, the apoptotic cell recognition signals are transferred to the Elmo–Dock1 complex to activate Rac1 upon engagement of Elmo by the cytoplasmic tail of BAI1. Activated Rac1 subsequently directs the engulfment of apoptotic cells by cytoskeletal rearrangement [2]. Also, BAI1 recognizes apoptotic myoblasts and facilitates the formation of myofibers by signaling through the Elmo–Dock1–Rac1 module [19]. Recently, Bai3, a mouse homolog of BAI1, was found to interact with Elmo and to promote myoblast fusion [4]. Thus, Elmo is involved in many cellular processes by modulating the activities of interacting proteins or by functioning as a scaffold.

Arhgef16 (Ephexin4) is a member of the Ephexin subfamily of Rho guanine nucleotide exchange factors. Five members of the Ephexin family have been identified so far but the details of their roles in various cellular processes are still being determined. However, Ephexin1 has been relatively well characterized in neuronal cells where it functions as a GEF for RhoA and is important for regulating axon guidance and spine morphogenesis via its interaction with EphA4 [20–22]. However, the roles of Ephexin-1, -2, -3 and -5 are less well understood although they are known to activate RhoA [20,21,23–25].

It was shown recently that Arhgef16 interacts with nucleotide-free RhoG or Rac1 but acts only as a RhoG-specific GEF, although it ultimately activates Rac1 via the RhoG–Elmo–Dock4 pathway. Also, Arhgef16 binds to the ephrin receptor A2 (EphA2) and promotes migration and invasion of breast cancer cells through a RhoG-dependent mechanism [26]. In addition, Arhgef16 renders cancer cells resistant to apoptosis by activating RhoG and PI3K downstream of EphA2 [26,27]. In contrast, the cellular function and biochemical properties of Ephexin4 are unknown in phagocytes during clearance of apoptotic cells.

We have identified Arhgef16 as an Elmo1-interacting protein from a yeast two-hybrid screen. Arhgef16 interacted with Elmo1 in yeast and mammalian cells and the possibility of a direct protein–protein interaction was demonstrated in vitro. Arhgef16 was capable of enhancing engulfment of apoptotic cells, and this enhanced removal of apoptotic cells was augmented by co-expression of Elmo1. The increased removal of apoptotic cells was the result of a synergy between Arhgef16 and Elmo1 in a RhoG-dependent and Dock1-independent manner. Here, we report a novel Elmo1-interacting protein, Arhgef16, capable of promoting removal of apoptotic cells.

2. Materials and methods

2.1. Cell cultures and transfections

293 T cells, J774 cells, MEF (mouse embryonic fibroblast), L cells, and primary astrocytes were maintained in DMEM supplemented with 10% FBS and 1% penicillin–streptomycin–glutamine, whereas LR73 cells were cultured in alpha-MEM with 10% FBS and 1% penicillin–streptomycin–glutamine. 293 T cells were transfected using the Profection mammalian transfection system (Promega) and the LR73 cells and L cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Nucleofection (Lonza) was used to introduce plasmids and siRNA (Dharmacon, smart pool) into J774 cells (kit V, program T-20), primary astrocytes (astrocyte kit, program T-20), and MEF (MEF2, program T-20).

2.2. Plasmids and antibodies

All plasmids generated in this study were sequenced to confirm their identity. The yeast clone N20 was used as a template to amplify smaller fragments of Arhgef16 and to generate FLAG-N20 and pEBG-N20. The mouse Arhgef16 cDNA, Ephexin-1 and Ephexin-2 cDNAs were purchased from Open Biosystems to construct pEBB-Arhgef16, pEBB-Arhgef16-FLAG, pEBB-Arhgef16-GFP, pEBB-Ephexin-1-FLAG, pEBB-Ephexin-2-FLAG and pGEX-4T-2-Arhgef16 by a PCR-based strategy in pEBB-FLAG, pEBB-GFP or pGEX-4T-2 vector. The Elmo1, Dock1, RhoG and Rac1 constructs used in this study have been described previously [2]. The antibodies used in the study were anti-FLAG (Sigma, M2), anti-GFP (Santa Cruz, B-2) and anti-GST (Santa Cruz, Z-5). The anti-Arhgef16 antibody and the anti-Elmo1 antibody were purchased from Proteintech and Bethyl Laboratories (A301-961A) and Santa Cruz Biotechnology (C-3), respectively.

2.3. Yeast two-hybrid screen

The yeast strain used in the two-hybrid screen was HF7C with His, Trp, and Leu as selection markers and the cDNA library was from a mouse 7-day embryo as described previously [2]. Yeast cells were transformed with the LiAc-based method. Before the library screen, it was confirmed that there was no transcriptional activation by the bait protein (full-length Elmo1). More than 10 million independent colonies were screened on the selective plate containing 5 mM 3-amino-1,2,4-triazole (3-AT) without His, Trp and Leu. The yeast clones growing on the selective plate were restreaked on the selective plate and grown into single colonies. The plasmid mixtures were then isolated from each clone after liquid culture of the yeast cells. The prey plasmids were rescued by transforming each plasmid mixtures into KC8 *Escherichia coli* and the rescued plasmids were re-transformed into HF7C yeast cells to confirm the specific interaction. After confirmation of the specific interaction, the prey vectors were sequenced to identify the inserts. Six final candidate genes putatively interacting with Elmo1 in yeast were identified from this screen.

2.4. Immunoblotting and immunoprecipitation

Primary cells or transfected cells were washed with ice-cold PBS and then lysed with a lysis buffer containing 50 mM Tris–Cl (pH 7.6), 150 mM NaCl, 10 mM NaPP, 10 mM NaF, 1 mM Na_3VO_4, 1% Triton X-100 and protease inhibitor cocktail. Proteins in the lysates were separated by SDS-PAGE, transferred onto a nitrocellulose membrane, and detected by appropriate antibodies. For the immunoprecipitation assays, primary cells, cell lines, or transiently transfected cells were washed with PBS and lysed. Lysates were incubated with protein-A/G-conjugated agarose beads and appropriate antibodies for 2 h. Finally, the agarose beads were extensively washed, proteins bound to agarose beads separated by SDS-PAGE, and transferred onto a nitrocellulose membrane. Then the immunoprecipitated or co-immunoprecipitated proteins were detected by immunoblotting.

2.5. Immunostaining

LR73 phagocytes transfected with the indicated plasmids were cultured on 18 mm Ø glass coverslips in a 12-well non-culture plate and incubated with fluorescent apoptotic thymocytes in a CO_2 incubator at 37 °C for 2 h. Afterwards, the cells was washed with ice-cold PBS, fixed with 4% paraformaldehyde in PBS for 15 min at room temperature, rinsed with PBS, and incubated with Hoechst 33342 (Invitrogen) in PBS for 5 min at room temperature. Images were acquired on Zeiss Axio Imager A1. NIH3T3 cells cultured on 18 mm Ø coverslips, inside a 12-well non-tissue-culture plate, were transfected, washed with ice-cold PBS and fixed with 4% paraformaldehyde in PBS for 15 min at RT. Then the cells were permeabilized with 0.1% PBST for 5 min and blocked with

blocking solution containing 3% BSA (BSA, BOVOGEN), 0.1% tritonX-100 (USB), 0.1% sodium azide (Sigma) and 1% HINGS (Gibco) in PBS for 30 min at room temperature. Next, the cells were incubated with primary antibody (anti-FLAG, Sigma) in 3% BSA in PBS at 4 °C overnight, washed with PBS and incubated with a secondary antibody (goat anti-mouse Ig conjugated to Alexa Fluor 555, invitrogen) for 1 h at room temperature. The cells were washed with PBS and stained with Hoechst 33342 (Invitrogen). Images were acquired using the Zeiss LSM 700 microscope.

2.6. Phagocytosis assay

1×10^5 LR73 cells, L cells, or MEF were transiently transfected with the indicated plasmids in a 24-well plate with Lipofectamine 2000 or nucleofection, respectively. One day after transfection, the cells were incubated with either 1.0 μl of 2 μm carboxylate beads, modified red fluorescence beads (Invitrogen), or fluorescent dye-stained apoptotic thymocytes in a CO_2 incubator at 37 °C for 2 h. For the generation of fluorescent apoptotic thymocytes, the thymi were acquired from 5–6 weeks old C57/Bl6 mice, cell clumps gently dissociated with a 5 ml syringe piston, and sieved to isolate individual thymocytes. Thymocytes were then stained with 25 μM of TAMRA-SE (Invitrogen) or 1 μM CypHer5e (GE Healthcare) in serum-free RPMI in a 5% CO_2 incubator at 37 °C for 20 min. Then the cells were destained in RPMI with 10% serum for 20 min, washed once and resuspended in RPMI with 10% serum and 1% penicillin–streptomycin–glutamine. Apoptosis of the thymocytes was induced using 50 μM of dexamethasone (calbiochem) in an incubator with 5% CO_2 at 37 °C for 4 h, and then washed with RPMI with 10% serum three times. Afterwards, apoptotic thymocytes were resuspended at a concentration of 5×10^5 cells in 300 μl of phagocyte culture medium and added to phagocytes and incubated in an incubator 5% CO_2 at 37 °C. Phagocytes were then washed with ice-cold PBS five times, trypsinized, and analyzed by flow cytometry. Phagocytes were identified by GFP fluorescence and ingested targets were identified by red (from TAMRA) or blue (from CypHer5E) fluorescence. The GFP- and red- or blue-fluorescent cells were considered to be phagocytes engulfing their targets. Most of the double-positive cells were phagocytes engulfing their targets or being engulfed themselves, but is not likely to be phagocytes loosely attached to their targets due to the extensive number of washes and trypsinization.

2.7. Purification of GST, GST-N20, GST-Arhgef16 and Elmo1 from bacteria

BL21 cells were transformed with pGEX-4T-2-Arhgef16, pGEX-4T-2-N20, or pGEX-4T-2 and induced with 1 mM IPTG. 4 h after induction, the cells were lysed and the lysates were incubated with glutathione-Sepharose beads for 4 h at 4 °C. The beads were then washed extensively and resuspended in washing buffer containing 20% glycerol. For purification of bacterially produced Elmo1, Elmo1 was purified using IMPACT (intein mediated purification with an affinity chitin-binding tag, NEB) system according to the manufacturer's instructions. Briefly, BL21 cells were transformed with pTYB21-Elmo1 and induced with 1 mM IPTG for 4 h and then the cells were lysed. The lysates were loaded onto a chitin column, washed with cleavage buffer containing 50 mM DTT, and finally Elmo1 was eluted. GST-tagged proteins and Elmo1 were mixed in 500 μl of a reaction buffer containing 20 mM Tris–Cl (pH 7.5), 200 mM NaCl and 1 mM EDTA and incubated for 2 h at 4 °C. After that, bound proteins were separated on SDS-PAGE, transferred onto nitrocellulose membrane and detected by western blot.

2.8. Quantitative RT-PCR

Total RNA from cell lines, tissues, and primary cells was isolated using the RNeasy kit (Qiagen) according the manufacturer's instructions and cDNA was synthesized from total RNA using Superscript III First-Strand Synthesis System (Invitrogen) based on the manufacturer's instructions. The relative levels of the transcripts were evaluated by the StepOnePlus real-time PCR system (Applied Biosystems) using the cDNA as the templates.

2.9. CRIB pull-down assay

293 T or LR73 cells were transfected with the indicated plasmids and then cultured for two or one day, respectively. Cell lysates from the transfected 293 T or LR73 cells were incubated with bacterially produced GST-CRIB proteins bound to glutathione-Sepharose beads for 1 h at 4 °C. Bound proteins on the beads were separated and detected using the Rac1-specific antibody.

2.10. Statistical analysis

All data are shown as the mean ± standard deviation. A two-tailed *t*-test was used to analyze statistical differences. Statistical significance was calculated using the GraphPad Prism 6 software and significance was assumed when p values were <0.05.

3. Results

3.1. Identification of Elmo1 interacting proteins

To identify novel Elmo1-interacting proteins, we performed a yeast two-hybrid screen using Elmo1 as bait. One candidate clone resulting from the screen was a fragment of Arhgef16, clone N20, containing amino acids 234 to 378 of Arhgef16 (Fig. 1A). Only yeast cells expressing both Elmo1 and the Arhgef16 fragment (N20) grew on the selective plate unlike yeasts transformed with only Elmo1 or the Arhgef16 fragment (Fig. 1B). To determine whether the interaction between Elmo1 and Arhgef16 could also be found in mammalian cells, FLAG-tagged Elmo1 and GST-tagged clone N20 were expressed in 293 T cells. GST-tagged N20 co-immunoprecipitated with FLAG-tagged Elmo1 (Fig. 1C), providing strong evidence that the fragment of Arhgef16 identified from the screen binds to Elmo1 in mammalian cells as well. In order to narrow the Elmo1 binding region within the N20 fragment, two smaller fragments, N20F (containing amino acids 234 to 287) and N20R (containing amino acids 288 to 378), were used for additional pull-down assays (Fig. 1A). Elmo1 specifically bound to N20R but not N20F (Fig. 1D); moreover, although the N20R fragment of Arhgef16 pulled down both the N-terminal and full-length Elmo1, it failed to pull down C-terminal Elmo1 (Fig. 1E). These data suggest that the N-terminus of Elmo1 interacts with Arhgef16.

3.2. Arhgef16 interacts directly with Elmo1

To confirm that full-length Arhgef16 is capable of binding Elmo1, GFP-tagged Arhgef16 and FLAG-tagged Elmo1 were co-expressed in 293 T cells. GFP-tagged Arhgef16 was found to co-immunoprecipitate with Elmo1 when FLAG-tagged Elmo1 was immunoprecipitated (Fig. 2A). Because over-expression of proteins could result in non-specific interactions, we also looked at the interactions between endogenous Elmo1 and either overexpressed or endogenous Arhgef16. Endogenous Elmo1 co-immunoprecipitated not only with overexpressed Arhgef16 in 293 T cells but also with endogenous Arhgef16 in HeLa cells (Fig. 2B and C), suggesting that Elmo1 binds to Arhgef16 at physiologically relevant protein levels. However, the weak interaction of Elmo1 with Arhgef16 at physiologically relevant levels was observed, which might be caused by the close proximity of the interaction site in Arhgef16 to the antibody (anti-Arhgef16) epitope (amino acids 175 to 225 of human Arhgef16). Analysis of images generated by confocal microscopy also supports our assertion that Elmo1 and Arhgef16 are interacting proteins. When Elmo1, Arhgef16, or both Elmo1 and Arhgef16 were expressed in NIH/3T3 cells, Elmo1 was found to be largely localized in the cytoplasm although it was sometimes detected faintly in the nucleus while Arhgef16 was

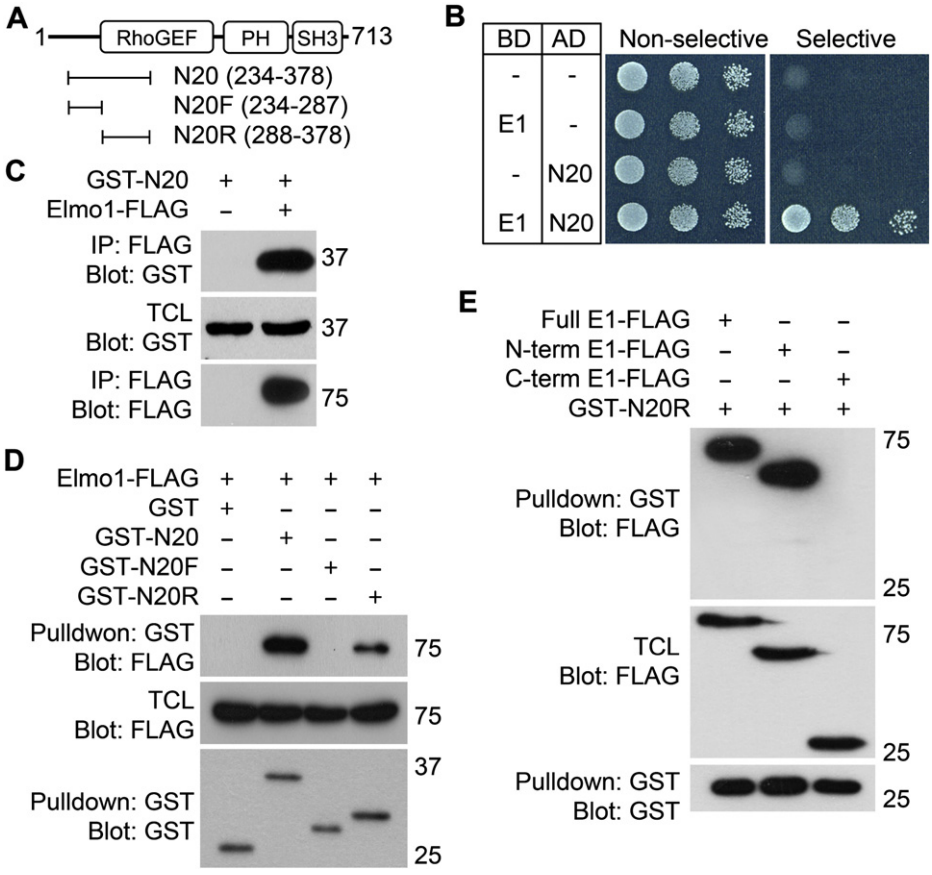

Fig. 1. Identification of an Elmo1 interacting protein from a yeast two-hybrid screen. A. Schematic diagram of Arhgef16. The clone identified in the yeast two-hybrid screen and the cloned fragments used in this study are indicated. B. Yeast cells transformed with the indicated plasmids were plated on a selective plate containing 5 mM 3-AT and deficient for histidine, leucine and tryptophan or on the non-selective plate deficient for leucine and tryptophan. E1, Elmo1. C, D, E. 293 T cells were transfected with the indicated plasmids, lysed, and the cell lysates were immunoprecipitated with the FLAG-specific antibody (C) or precipitated with glutathione Sepharose beads (D, E). Co-immunoprecipitated proteins (C) or co-precipitated proteins (D, E) were resolved by SDS-PAGE and detected with the GST-specific antibody (C) or the FLAG-specific antibody (D, E). TCL, total cell lysate; and E1, Elmo1.

expressed in the cytoplasm and nucleus. When Elmo1 and Arhgef16 were co-expressed, the two proteins co-localized distinctly in the cytoplasm (Fig. 2D).

Because these assays could not demonstrate direct interaction of the proteins, we performed in vitro binding assays to determine whether the interaction is direct or possibly mediated by a third protein. Recombinant GST-N20, GST-Arhgef16 and Elmo1 were expressed and purified from bacteria and in vitro pull-down assay was performed using glutathione-conjugated agarose beads. Both GST-N20 and GST-Arhgef16 pulled down Elmo1, albeit with different binding strengths, but GST alone failed to precipitate Elmo1 (Fig. 2E). These binding assays provide evidence that Elmo1 and Arhgef16 interact directly. Together, results from these in vitro pull-down assays and the data from yeast, where neither Elmo nor Arhgef16 homologs exist, suggest that the interaction between Elmo1 and Arhgef16 is a direct protein–protein interaction.

The functional domains of Arhgef16, especially the DH domain, are well conserved among members of the Ephexin family. We found that N20R, containing a part of the DH domain of Arhgef16, binds to Elmo1 and this raised the possibility that Elmo1 interacts with the other Ephexins. Therefore, we cloned Ephexin-1 and Ephexin-2 to determine whether Elmo1 could interact with them. Surprisingly, Elmo1 also interacted with Ephexin-1 and Ephexin-2 although they associated with Elmo1 relatively weakly compared to Arhgef16 (Fig. 2F). These results are interesting because other members of the Ephexin family are known to activate RhoA [20–22,24,25]. In addition, we tested the interaction between Arhgef16 and Elmo homologs. Interestingly, Elmo2 interacted with Arhgef16 much more strongly than Elmo1 but we could detect no interaction between Elmo3 and Arhgef16 (Fig. 2G). These data suggest that Elmo may function as a general modulator for members of the Ephexin family.

3.3. Arhgef16 promotes the engulfment of apoptotic cells

It is known that Elmo functions with Dock1 as a bipartite GEF to activate Rac1 and that Elmo is involved in the phagocytosis of apoptotic cells by Rac1-mediated actin cytoskeleton reorganization. Because Elmo is important for clearing apoptotic cells, we examined the role of Arhgef16 during phagocytosis of two types of targets, carboxylate-modified beads mimicking apoptotic cells or apoptotic thymocytes. Arhgef16 over-expression in LR73 phagocytes promoted engulfment of 2 μm carboxylate-modified beads compared to the GFP control, and engulfment of the beads was further increased in cells expressing greater amounts of Arhgef16 (Fig. 3A). Analysis by fluorescence microscopy also confirmed that Arhgef16 promotes engulfment of apoptotic cells. A higher frequency of LR73 phagocytes expressing Arhgef16 contained apoptotic bodies compared to a GFP control (Fig. 3B).

To demonstrate that the increase in engulfment of apoptotic cells mediated by Arhgef16 was caused by increased internalization rather than increased binding of the targets, two parallel experiments were performed. First, images generated by confocal microscopy confirmed apoptotic bodies inside phagocytes in Arhgef16-expressing cells rather than simply being attached to the phagocytes (Fig. 3C). Second, to focus on phagocytes with completely internalized apoptotic cells, the apoptotic cells were stained with CypHer5E, a pH-sensitive dye whose fluorescence increases when apoptotic cells enter the acidic environment of

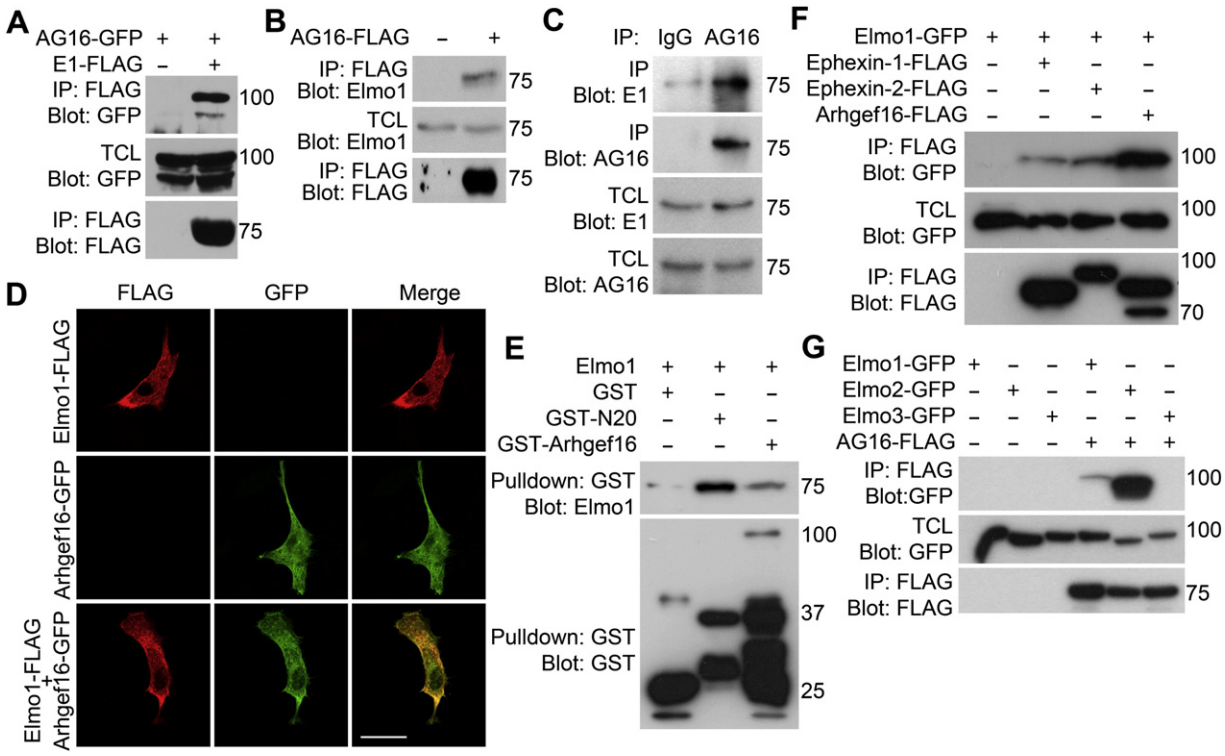

Fig. 2. Arhgef16 directly interacts with Elmo1. A, B. 293 T cells were transfected with the indicated plasmids, lysed, and the cell lysates were immunoprecipitated with anti-FLAG antibody. Co-immunoprecipitated proteins were detected with the GFP-specific (A) or Elmo1-specific antibodies (B). E1, Elmo1; and TCL, total cell lysate. C. HeLa cells were lysed and Arhgef16 was immunoprecipitated with anti-Arhgef16. Co-immunoprecipitated proteins were detected with the Elmo1-specific antibody. D. NIH/3T3 cells were transfected with the indicated plasmids and stained with anti-FLAG antibody. Scale bar, 30 μm. E. GST, GST-N20, GST-Arhgef16 and Elmo1 were purified from bacteria, incubated in a reaction buffer for 2 h, and precipitated with glutathione Sepharose beads. The precipitates were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and detected by immunoblot analysis. F, G. 293 T cells were transfected with the indicated plasmids, lysed, and the cell lysates were immunoprecipitated with anti-FLAG antibody. Co-immunoprecipitated proteins were detected by anti-GFP antibody.

the phagolysosome. Arhgef16 overexpressing phagocytes showed a higher percentage of CypHer5E-positive cells compared to a GFP control (Fig. 3D). Moreover, analysis by flow cytometry suggests that Arhgef16 facilitates engulfment of apoptotic cells by internalization because unbound or loosely bound apoptotic cells are removed during the preparation of samples due to repeated washes and trypsinization. These data support the notion that Arhgef16 promotes engulfment of apoptotic cells through increased internalization of apoptotic bodies.

Clearance of apoptotic cells is an energy-dependent process that requires actin cytoskeleton rearrangement and "dragging" forces inside the cells, generated by myosin-II, to completely internalize the apoptotic bodies. To address whether enhanced removal of apoptotic cells by Arhgef16 is also governed by the engulfment machinery, LR73 phagocytes expressing Arhgef16 with apoptotic cells were incubated with cytochalasin D (an inhibitor of actin polymerization), ML-7 (an inhibitor of myosin light chain kinase), sodium azide (an inhibitor of ATP synthesis), or by incubating the cells at 4 °C (to inhibit ATP-dependent cellular processes). Interestingly, Arhgef16-induced engulfment of apoptotic cells was completely abrogated by these treatments (Fig. 3E), indicating that the increased uptake of apoptotic cells mediated by Arhgef16 depends on the generally known cytoskeletal engulfment machinery.

The expression of Arhgef16 is not ubiquitous but is confined to certain tissues and cell types. For example, *Arhgef16* transcripts were detected predominately in thymic epithelial cells and astrocytes (Fig. 3F). To verify that knockdown of Arhgef16 results in loss of function, primary astrocytes expressing Arhgef16 endogenously were transfected with *Arhgef16* siRNA or a control siRNA. Expression of *Arhgef16* transcripts in *Arhgef16* siRNA-transfected cells was reduced by up to 60% compared to the control experiment (Fig. 3G) and engulfment of apoptotic cells in *Arhgef16* siRNA-transfected cells was lower than in control siRNA-transfected cells (Fig. 3H). Collectively, these data suggest that enhanced engulfment of targets mediated by Arhgef16 results from increased internalization of targets, which is an ATP-dependent process and requires cytoskeleton rearrangement.

3.4. Elmo is necessary for Arhgef16 function

Next, we determined whether Elmo1 is necessary for Arhgef16-mediated engulfment of apoptotic targets. To address this possibility, LR73 phagocytes expressing Arhgef16, Elmo1, or both Arhgef16 and Elmo1 were incubated with surrogate targets or apoptotic cells. Elmo1 expression in LR73 cells did not promote engulfment of either carboxylate-modified beads or apoptotic thymocytes. In contrast, expression of Arhgef16 in LR73 cells increased the percentage of cells ingesting surrogate targets or apoptotic cells. Interestingly, when Arhgef16 and Elmo1 were expressed together in LR73 cells, there was a synergistic increase in engulfment of both types of targets (Fig. 4A and B). In order to test further the necessity of Elmo1 for Arhgef16 function, a slightly different strategy was used. When Elmo1 was depleted and Arhgef16 was expressed (Fig. 4C), Arhgef16 expressing J774 cells surprisingly failed to increase uptake of the beads or apoptotic cells. However, J774 cells expressing both Elmo1 and Arhgef16 showed substantially higher levels of engulfed carboxylate beads or apoptotic thymocytes (Fig. 4D and E). These results provide unequivocal evidence that Elmo1 is necessary for Arhgef16-mediated engulfment of apoptotic cells.

To demonstrate the specificity and relevance of the interaction between Arhgef16 and Elmo1 during clearance of apoptotic cells, we performed additional binding and competition assays. Our yeast two-hybrid data and immunoprecipitation assays in mammalian cells showed that Arhgef16 clone N20 binds to Elmo1 more vigorously than

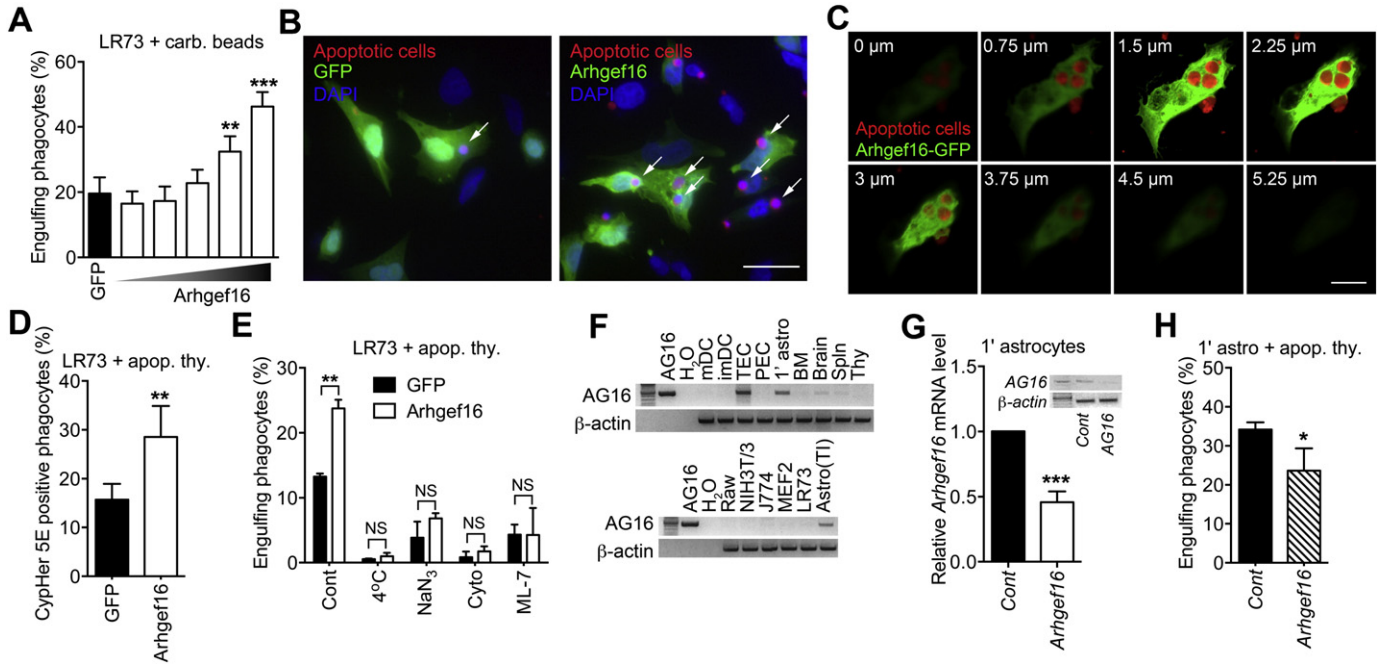

Fig. 3. Arhgef16 promotes engulfment of apoptotic cells. A. LR73 phagocytes transfected with GFP or various amounts of Arhgef16 were incubated with 1 μl of red fluorescent carboxylate-modified bead (2 μm in diameter) for 1 h, 1 day after transfection. The cells were extensively washed with ice-cold PBS, trypsinized, and analyzed by flow cytometry. Red fluorescence-positive cells, in cells expressing equivalent levels of proteins gated on the basis of GFP expression, were considered to be phagocytes engulfing the beads. B. LR73 phagocytes transfected with GFP (left) or Arhgef16 and GFP (right) were incubated with TAMRA-labeled apoptotic thymocytes for 2 h, stained, and analyzed by fluorescence microscopy. Arrows indicate engulfed apoptotic cells. Scale bar, 10 μm. C. LR73 phagocytes expressing Arhgef16-GFP were incubated with TAMRA-stained apoptotic thymocytes for 2 h, stained, and analyzed by confocal microscopy. The numbers indicate the height from bottom set in the microscope. Scale bar, 10 μm. D. CypHer5E-stained apoptotic thymocytes were incubated with LR73 cells transfected with GFP or Arhgef16-GFP for 2 h. Afterwards, phagocytes were analyzed by flow cytometry. GFP and CypHer5E double-positive phagocytes were considered to be phagocytes internalizing apoptotic cells. E. LR73 phagocytes transfected with either GFP alone or GFP and Arhgef16 were incubated with TAMRA-stained apoptotic thymocytes in the presence of NaN_3 (1 mM), cytochalasin D (1 μM), ML-7 (30 μM), or at 4 °C. Phagocytes engulfing targets were analyzed by flow cytometry. Cont, control; Cyto, cytochalasin D; and NS, not significant. F. cDNA was synthesized from the indicated cell lines or tissues and then the *Arhgef16* transcripts were amplified by polymerase chain reaction. mDC, mature dendritic cells; iDC, immature dendritic cells; TEC, thymic epithelial cells; PEC, peritoneal exudate cells; astro, astrocytes; BM, bone marrow; Spln, spleen; and Thy, thymus. G. Total RNA from primary astrocytes nucleofected with control siRNA or *Arhgef16*-specific siRNA was extracted, cDNA was synthesized, and *Arhgef16* transcripts were detected by qRT-PCR. Inset, *Arhgef16* transcripts from the nucleofected cells were amplified using conventional PCR. Cont, control; AG16, Arhgef16. H. Primary astrocytes transfected with control siRNA or *Arhgef16*-specific siRNA were incubated with TAMRA-stained apoptotic thymocytes and the cells engulfing apoptotic thymocytes were evaluated by flow cytometry. Data are shown as the mean ± standard deviation and are representative of at least three independent experiments. *P < 0.05, **P < 0.01, and ***P < 0.001.

does the full-length Arhgef16. Thus, we tested whether the N20 fragment might block binding of full-length Arhgef16 to Elmo1. Interestingly, the N20 fragment competed with full-length Arhgef16 for binding to Elmo1, that is, it inhibited the interaction between Elmo1 and full-length Arhgef16 (Fig. 4F). More intriguingly, expression of the Arhgef16 fragment severely inhibited engulfment of apoptotic cells in a dose-dependent manner in primary astrocytes expressing Arhgef16 endogenously (Fig. 4G). In addition, expression of the Arhgef16 N20 fragment in LR73 phagocytes overexpressing Elmo1 and Arhgef16 disrupted the synergistic effect of the two proteins and also inhibited the ability of Arhgef16 to remove apoptotic cells (Fig. 4H). These data suggest that Elmo1 is necessary and its interaction with Arhgef16 is required for Arhgef16-mediated uptake of apoptotic cells.

3.5. Arhgef16 promotes engulfment of apoptotic cells in a Dock1-independent and RhoG-dependent manner

Arhgef16 is a guanine nucleotide exchange factor for the Rho family of GTPases and it is known to be a RhoG-specific GEF that can activate Rac1 through the RhoG–Elmo–Dock4 pathway [26]. Because the activation of Rac1 is a critical step in the removal of apoptotic cells and it is known that Arhgef16 can activate Rac1 through the activation of RhoG, enhanced apoptotic cell clearance by Arhgef16 may be also mediated by the activation of Rac1. To test this possibility, we used a CRIB pull-down assay to measure the levels of activated (or GTP-bound) Rac1 in Arhgef16 overexpressing cells. Expression of Arhgef16 in LR73 cells resulted in increased activation of Rac1 (1.5 fold increase) compared to mock-transfected cells. More interestingly, and consistent with our previous observations, the activation of Rac1 in cells overexpressing Arhgef16 was more prominent when Elmo1 was co-expressed (2.4 fold increase) (Fig. 5A). Next, we used a dominant-negative form of Rac1 (RacN) to further evaluate the dependency of Arhgef16-mediated engulfment on Rac1. Co-expression of RacN and Arhgef16 effectively negated any increases in uptake of apoptotic cells mediated by Arhgef16 (Fig. 5B). These results strongly suggest that Rac1 is activated in phagocytes overexpressing Arhgef16, which results in increased uptake of apoptotic cells. Also, the synergy between Elmo1 and Arhgef16 that allows increased clearance of apoptotic cells results from an incremental increase in Rac1 activation.

Dock1 is a well-known Elmo binding protein and activates Rac1 during the engulfment of apoptotic cells. Thus, we examined whether Dock1 is necessary for Arhgef16-induced engulfment of apoptotic cells. First, expression of Dock1-ISP, a dominant-negative form of Dock1 unable to bind to Rac1, strongly inhibited a basal level of engulfment of apoptotic cells. Surprisingly, co-expression of Dock1-ISP and Arhgef16 in LR73 phagocytes showed minimally impaired engulfment compared to cells expressing only Dock1-ISP (Fig. 5C). Second, to test the effect of Dock1 depletion on Arhgef16-mediated engulfment, *Dock1* expression was reduced by *Dock1*-specific siRNA in MEF and L cells. Decreasing *Dock1* transcript levels had no effect on Arhgef16-mediated engulfment of apoptotic cells in MEF (Fig. 5D and E) and L cells (Fig. 5F and G) although approximately 80% of the *Dock1* transcripts were depleted by the siRNA treatment.

It has been proposed that Arhgef16 is a RhoG-specific GEF that transduces signals to downstream molecules through Elmo and Dock4. Thus,

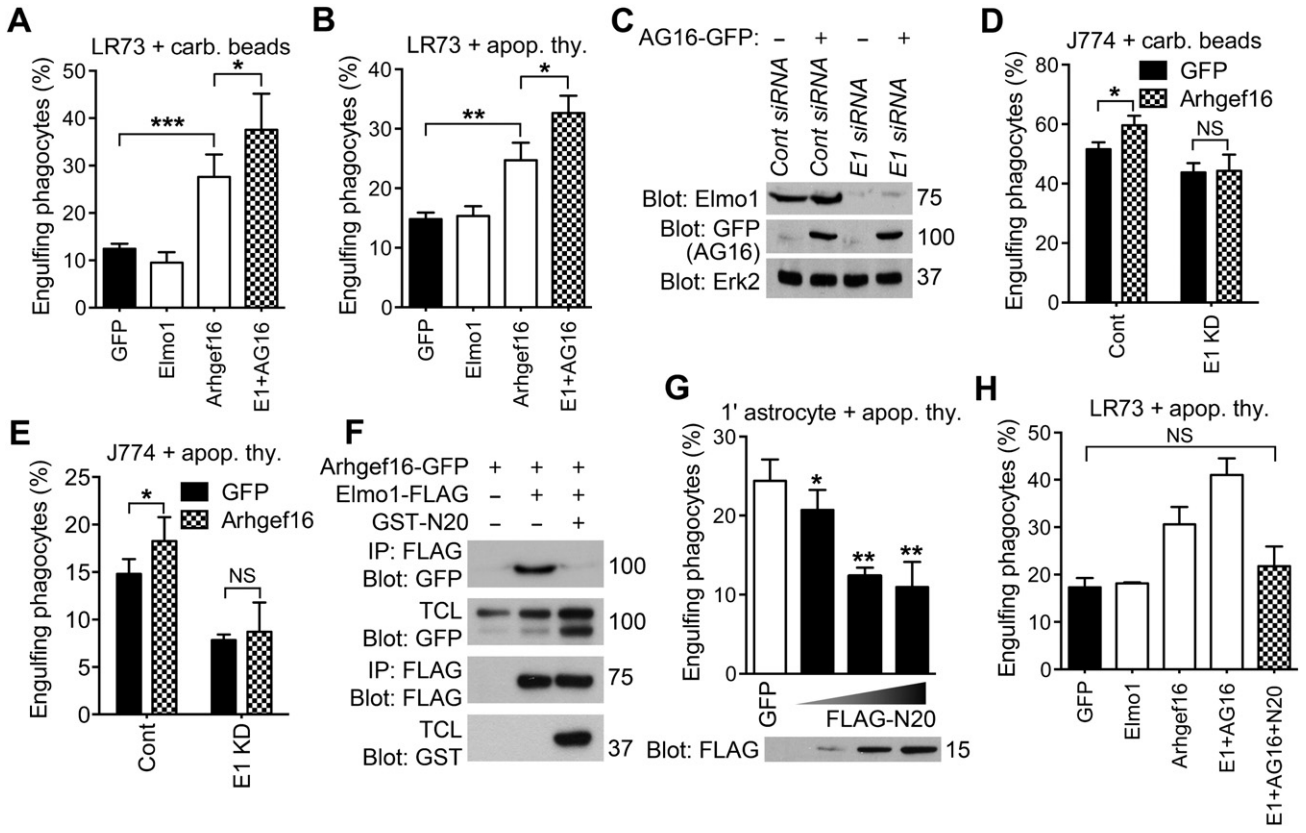

Fig. 4. Elmo is required for Arhgef16-mediated engulfment of apoptotic cells. A, B. LR73 phagocytes were transfected with the indicated plasmids and then incubated with red fluorescent carboxylate-modified beads (A) or TAMRA-stained apoptotic thymocytes (B) for 2 h and then subjected to flow cytometry. Cells positive for GFP and red fluorescence were considered to be phagocytes engulfing their targets. E1, Elmo1; AG16, Arhgef16. C. J774 cells stably expressing control or *Elmo1* siRNA were transfected with Arhgef16, lysed, and immunoblotted. Cont, control; E1, Elmo1; and AG16, Arhgef16. D, E. J774 cells stably expressing control or *Elmo1* siRNA were transfected with GFP or Arhgef16 and GFP, and incubated with red fluorescent carboxylate-modified beads (D) or TAMRA-stained apoptotic thymocytes (E). Flow cytometry was used to gate GFP and red fluorescent double-positive cells which were phagocytes ingesting targets. NS, not significant. F. 293 T cells were transfected with the indicated plasmids and immunoprecipitation assay was performed with anti-FLAG antibody. Co-immunoprecipitated proteins were detected with anti-GFP antibody. G. Primary astrocytes expressing different amounts of the Arhgef16 fragment (N20) interacting with Elmo1 were incubated with TAMRA-stained apoptotic thymocytes and analyzed by flow cytometry. Expression levels of the Arhgef16 fragment were detected by immunoblot analysis and shown at the bottom. H. The indicated plasmids were introduced into LR73 cells, and then the cells were incubated with TAMRA-stained apoptotic thymocytes. Flow cytometry analysis was used to evaluate phagocytes ingesting apoptotic cells. E1, Elmo1; AG16, Arhgef16; and NS, not significant. Data are shown as the mean ± standard deviation and are representative of at least three independent experiments. *$P < 0.05$, and **$P < 0.01$.

we also investigated the possibility that RhoG relays signals generated by Arhgef16 to downstream molecules during clearance of apoptotic cells. RhoGN, a dominant-negative form of RhoG, lowered basal uptake levels of apoptotic cells in LR73 cells. More interestingly, the presence of RhoGN in Arhgef16-expressing cells completely blocked Arhgef16-mediated engulfment of apoptotic cells (Fig. 6A). To further evaluate the role of RhoG during apoptotic cell clearance mediated by Arhgef16, expression of *RhoG* was knocked-down using *RhoG*-specific siRNA. *RhoG* transcript levels in these siRNA transfected MEFs were about 70% less than control siRNA transfected MEFs (Fig. 6B). Intriguingly, Arhgef16 could not facilitate engulfment of surrogate targets or apoptotic cells when RhoG was depleted (Fig. 6C and D). This same experiment was repeated with L cells and RhoG transcript levels were also effectively reduced by the siRNA treatment (Fig. 6E), which suppressed Arhgef16-induced engulfment of surrogate targets (Fig. 6F). Taken together, these results suggest that Arhgef16 mediates engulfment of apoptotic cells in a Dock1-independent and RhoG-dependent manner and it consists of a parallel pathway from Dock1 for the activation of Rac1 during engulfment of apoptotic cells.

4. Discussion

Removal of apoptotic cell is an essential biological process in multicellular organisms during development, for maintaining tissue homeostasis, and for immune system regulation. Genetic studies using *C. elegans* as a model organism have helped to identify several genes involved in the clearance of cell corpses. CED-12 was originally identified from these studies and it functions together with CED-5 to activate CED-10 [11,28,29]. Mammalian homologs of CED-12, CED-5, and CED-10 are Elmo, Dock1, and Rac1, respectively, and it is known that Elmo is the regulatory component of the Elmo-Dock1 bipartite GEF complex responsible for activating Rac1 [3,11,30].

Elmo is an intriguing protein that modulates the activities of other proteins although it does not have intrinsic catalytic activity. Thus far, various proteins including a plasma membrane protein and a nuclear protein have been identified as Elmo-interacting proteins [1,2,7,31]. However, there might be other unidentified proteins whose activities are regulated directly by Elmo1. Therefore, we performed a yeast two-hybrid screen to search for Elmo1-binding proteins and to determine whether their activities are modulated by Elmo1. We have identified six new candidate proteins that interact with Elmo1, apart from previously identified and characterized Elmo1 binding partners including RhoG and Dock1. One candidate from our screen was Arhgef16 and we verified the interaction between Elmo1 and Arhgef16 in mammalian cells; furthermore, this interaction was direct (protein-to-protein) as determined by in vitro binding assays.

Over-expression of Arhgef16 in phagocytes promoted engulfment of apoptotic cells and co-expression of Elmo1 and Arhgef16 synergistically increased engulfment of targets such as surrogate beads and apoptotic cells. In contrast, depletion of Elmo1 abrogated the enhanced uptake

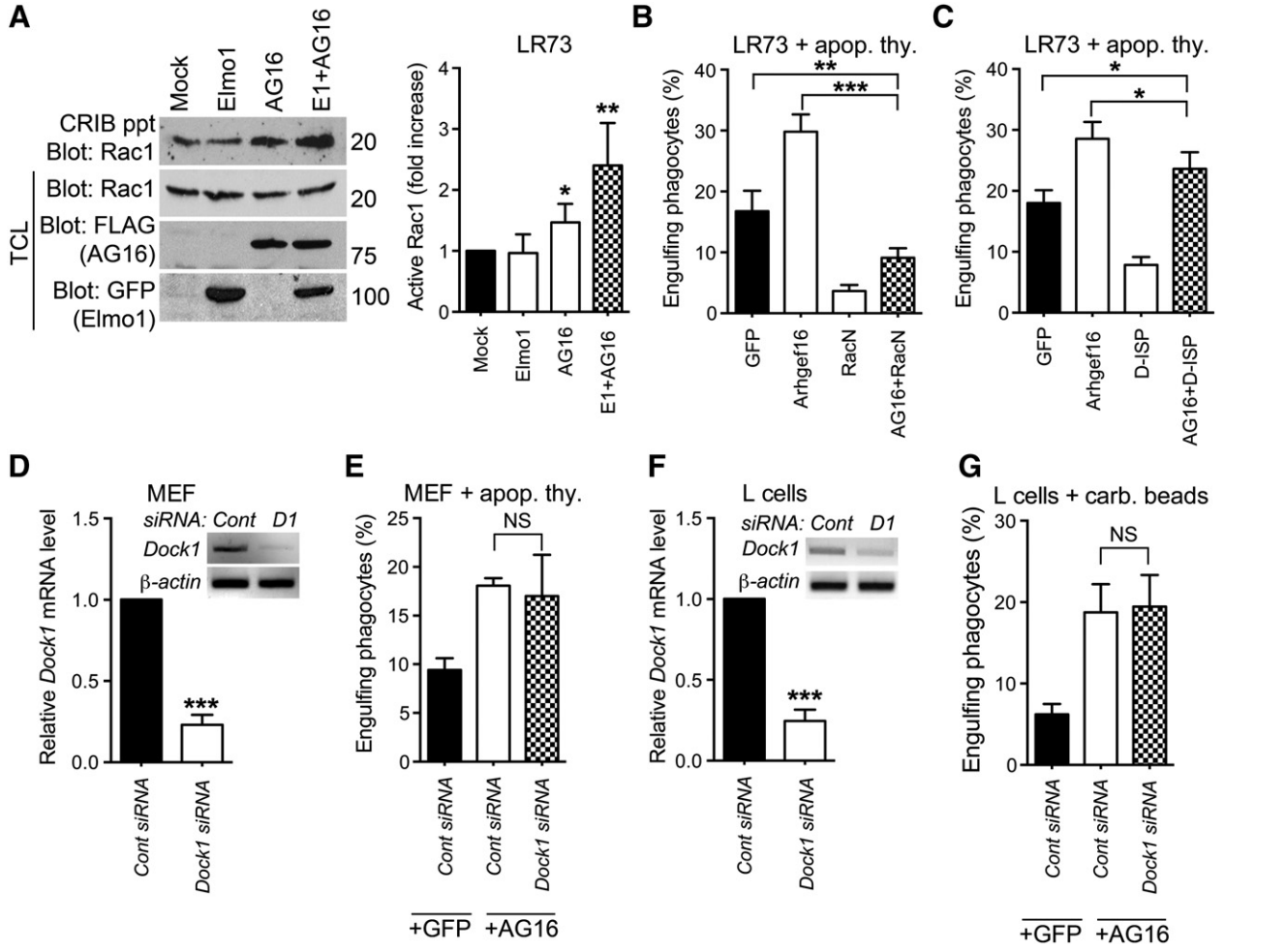

Fig. 5. Arhgef16 promotes engulfment of apoptotic cells in a Rac1-dependent manner. A. LR73 cells were transiently transfected with the indicated plasmids. 1 day after transfection, the cells were lysed. GTP-Rac1 was precipitated with GST-CRIB and detected by immunoblot analysis. A representative experiment is shown (left). Active Rac1 levels in the transfected cells were quantified using the ImageJ program (right). TCL, total cell lysate. AG16, Arhgef16, E1, Elmo1. B, C. LR73 cells expressing indicated proteins were incubated with TAMRA-stained apoptotic thymocytes and analyzed by flow cytometry. AG16, Arhgef16; D-ISP, Dock1-ISP. D, MEF cells were nucleofected with control siRNA or *Dock1*-specific siRNA. One day after nucleofection, total RNA from the nucleofected cells was extracted, cDNA was synthesized, and *Dock1* transcripts were detected using qRT-PCR. Inset, *Dock1* transcripts from siRNA nucleofected cells were amplified by conventional PCR. E, MEF cells nucleofected with the indicated siRNA and plasmids were incubated with TAMRA-stained apoptotic cells and analyzed by flow cytometry. F, L cells were transfected with the indicated siRNA, and *Dock1* transcript levels were detected by qRT-PCR or conventional PCR (inset). G. L cells transfected with the indicated siRNA and plasmids were subjected to an engulfment assay. Data are shown as the mean ± standard deviation and are representative of at least three independent experiments. *P < 0.05, **P < 0.01, and ***P < 0.001.

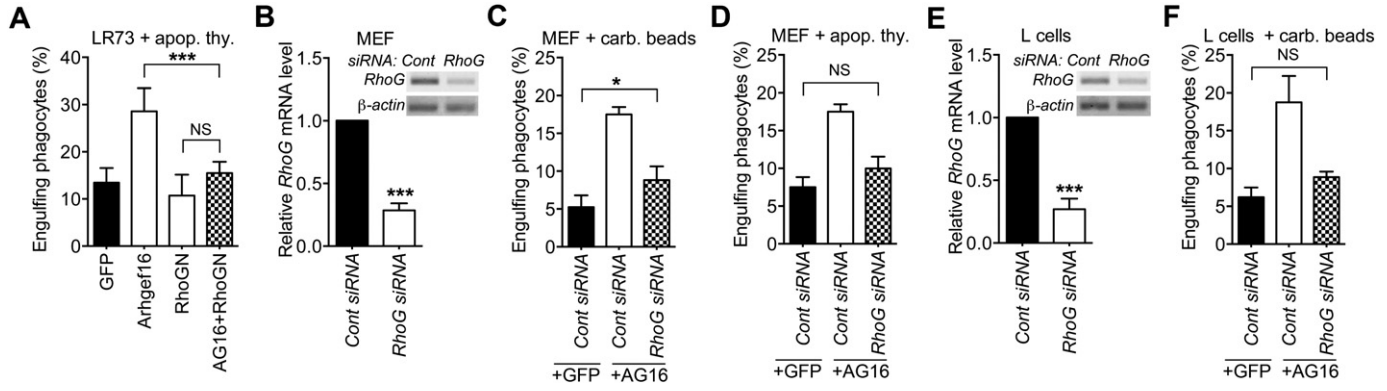

Fig. 6. RhoG mediates Arhgef16-promoted engulfment of apoptotic cells. A. LR73 cells were transfected with the indicated plasmids, incubated with TAMRA-stained apoptotic cells, and analyzed by flow cytometry. GFP and TAMRA double-positive phagocytes were considered to be phagocytes ingesting targets. B. MEF cells were nucleofected with control or *RhoG*-specific siRNA, and cDNA was synthesized from total RNA extracted from the nucleofected cells. *RhoG* transcript levels were detected by qRT-PCR or conventional PCR (inset). C, D. MEF cells nucleofected with the indicated siRNA or plasmids were incubated with carboxylate beads (C) or TAMRA-stained apoptotic cells (D). After incubation, phagocytosis of targets was evaluated using flow cytometry. E. L cells were transfected with control or *RhoG*-specific siRNA. *RhoG* messages were detected by qRT-PCR or conventional PCR (inset). F. L cells were transfected with the indicated plasmids and siRNA and then incubated with carboxylate beads. Engulfment of surrogate targets by phagocytes was measured by flow cytometry. Data are shown as the mean ± standard deviation and are representative of at least three independent experiments. *P < 0.05, and ***P < 0.001.

of apoptotic cells by Arhgef16. Interestingly, enhanced engulfment by Arhgef16 was linked to the activation of Rac1, and Rac1 activation was significantly greater when Elmo1 was co-expressed with Arhgef16. These observations bear a striking resemblance to the mechanism used by Dock1 to activate Rac1, with a key difference being that Dock1 itself has intrinsic GEF activity for Rac1. Here, Elmo helps to relieve of the self-inhibitory state of Dock1, thereby stabilizing nucleotide-free Rac, and allowing Dock1 to access Rac1 [12,14,15,32]. In contrast, Arhgef16 is a RhoG-specific GEF and it is thought that Arhgef16 activates Rac1 by utilizing the RhoG–Elmo–Dock4 pathway [26]. Our preliminary observations suggest that the SH3 domain of Arhgef16 binds to Elmo as well as to itself, as does the amino-terminal SH3 domain of Dock1 (unpublished data). Thus, Elmo may regulate the GEF activity of Arhgef16 by relieving the self-inhibitory state of Arhgef16, as it does for Dock1. This would allow RhoG to become activated by Arhgef16, followed by Rac1 activation. This might be a general mechanism whereby Elmo alleviates inhibitory intramolecular interactions, thereby helping to regulate the activities of Elmo-interacting GEFs. This will be an interesting subject for future study.

Also, we found that *Arhgef16* transcripts were restricted to certain cell lines or tissues, especially the brain or cell lines derived from the brain. However, Dock1 is ubiquitously expressed. The different expression patterns of Dock1 and Arhgef16 might explain why Rac1 is activated directly or indirectly by two different GEFs during engulfment of apoptotic cells. It is plausible that Dock1 is the major GEF for Rac1 in most phagocytes including professional phagocytes such as macrophage and dendritic cells whereas Arhgef16 is specialized to function mainly in non-professional phagocytes such as astrocytes where Arhgef16 transcripts are plentiful. In support of this view, it has been reported that astrocytes are highly phagocytic cells capable of clearing apoptotic cells, and that Elmo and Arhgef16 are highly expressed in astrocytes [2,33,34]. Additionally, one interesting finding of our study is that Arhgef16-induced engulfment of apoptotic cells is Dock1-independent although Rac1 becomes activated. Thus, it is interesting to learn of how Rac1 becomes activated. Previously, it was reported that Arhgef16 might activate Rac1 through Dock4 [26]. However, we found that Dock4 is not expressed in MEF or L cells used in the study. Instead, Dock3 and Dock5 are expressed (data not shown). Thus, it is plausible that the activation of Arhgef16 is channeled into Dock3 or Dock5 to activate Rac1 at least in these phagocytes, MEF or L cells.

The protein just upstream of Arhgef16 and presumably necessary for regulating engulfment of apoptotic cells is unknown. Previously, it was shown that Arhgef16 binds to the ephrin receptor A2 (EphA2). However, it is not known whether EphA2 or another unidentified protein functions as the receptor upstream of Arhgef16 for apoptotic cells. BAI1, an apoptotic cell receptor, is expressed highly in the brain and astrocytes and also interacts with Elmo. Thus, BAI1 is also a candidate receptor that could function just upstream of Arhgef16. It will be interesting to determine what proteins are upstream of Arhgef16 and involved in the removal of apoptotic cells.

The evidence presented here suggests that Arhgef16 is an Elmo1-binding protein. Moreover, Arhgef16 activates Rac1 in a RhoG-dependent and Dock1-independent manner and there is greater Rac1 activation in the presence of both Arhgef16 and Elmo1. Cooperation between Arhgef16 and Elmo1 may help to activate RhoG resulting in the activation of Rac1, which results in a synergistic increase in engulfment of apoptotic cells. However, the mechanism by which Elmo helps Arhgef16 activate RhoG needs to be clarified in future studies. Ultimately, the knowledge gained from these studies may be harnessed to develop cures for diseases resulting from defects of removal of apoptotic cells.

Acknowledgments

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